FEBS Letters 582 (2008) 2931–2936

A halorhodopsin-overproducing mutant isolated from an extremely haloalkaliphilic archaeon *Natronomonas pharaonis*

Kunio Ihara[a,*], Akihiro Narusawa[b], Keita Maruyama[b], Masashi Takeguchi[b], Tsutomu Kouyama[b]

[a] *Center for Gene Research, Nagoya University, Chikusa-ku Furo-cho 1, Nagoya, Japan*
[b] *Graduate School of Science, Nagoya University, Chikusa-ku Furo-cho 1, Nagoya, Japan*

Received 16 May 2008; revised 21 June 2008; accepted 18 July 2008

Available online 29 July 2008

Edited by Richard Cogdell

Abstract The halorhodopsin (hR)-overproducing mutant strain KM-1 was isolated from the extremely haloalkaliphilic archaeon *Natronomonas pharaonis* type strain DSM2160^T. hR-enriched membranes were easily obtained by washing the cells with distilled water. The membranes were claret colored owing to two pigments: hR and bacterioruberin. The hR component in the absorption spectra changed from blue to purple upon the addition of Cl^- and had a K_m value of 1.7 mM. Overexpression of hR in strain KM-1 might be caused by the point mutation Asp324 → Asn in the bacteriorhodopsin activator homologues of *N. pharaonis*. The mutation changed the hR-expression pattern from inducible to constitutive in the late exponential phase.

Keywords: Halorhodopsin; Haloalkaliphiles; Bacterioruberin; Bacteriorhodopsin activator; Chloride ion; *Natronomonas pharaonis*

1. Introduction

Natronomonas pharaonis (formerly known as *Natronobacterium pharaonis*) is an extremely haloalkaliphilic archaeon, which was originally isolated from alkaline salt lakes in Egypt (Soda Lake) [1] and Kenya [2]. This archaeon grows optimally in environments containing approximately 3.5 M NaCl at pH 8.5–9.0 and retains its rod-shaped morphology even at submillimolar magnesium ion concentrations. Recent genome analysis of the type strain of *N. pharaonis* (DSM 2160^T) revealed that this strain was adapted to cope with the severe ammonia and heavy metal deficiencies that occur at high pH levels [3]. In this archaeon, multiple cell surface glycoproteins probably form a protective complex cell envelope. These archaea have been reported to possess peculiar membranes that contain C_{20}–C_{25} in addition to C_{20}–C_{20} diether core lipids [2] and to lack detectable amounts of glycolipids [2].

N. pharaonis contains two archaeal-type rhodopsins – halorhodopsin (hR) [4] and phoborhodopsin (pR; also known as sensory rhodopsin II) [5]. The former functions as a light-driven chloride ion pump [4], and the latter, as a sensor for photophobic responses against blue light [6]. Both rhodopsins were investigated in detail in the strain SP1 (DSM 3395^T). The identity indices of the amino acid sequences of hR and pR were 96% and 97%, respectively, between the strains DSM 2160^T and DSM 3395^T (see Supplement for details). Unlike the halophilic archaeon *Halobacterium salinarum* (*H. salinarum*), which thrives at neutral pH, the archaeon *N. pharaonis* lacks two rhodopsins, namely, bacteriorhodopsin (bR) and sensory rhodopsin (sR) [3].

In 1986, Bivin and Stoeckenius reported a carotenoid-less spontaneous mutant of haloalkaliphilic archaea and suggested the presence of hR and pR in that haloalkaliphilic archaeon by using membranes from the mutant strain [7]. They estimated that the archaeon contained 6000 molecules of hR, which is not a very large number in comparison with the 20000 molecules observed in the *H. salinarum* strain JW-12 [7]. In the natural hR overproducing mutant like L33, its expression level was limited at most several times higher than that of the wild-type strain [8], and only recombination system using bacterioopsin (*bop*) promoter could express hR in amount compatible to the bR level [9]. This recombinant strain, however, was unstable, and the direction of the expressed hR in its membrane differed from that of physiologically expressed hR [10].

In this paper, we report the isolation and characterization of the hR-overproducing mutant strain KM-1 of the haloalkaliphilic archaeon *N. pharaonis*. The hR level in strain KM-1 was several tens of times greater than that in the wild-type, and its transcription regulation changed from the inducible pattern in the wild-type to the constitutive pattern. We detected a point mutation in a bacteriorhodopsin activator (*bat*)-homologous gene in strain KM-1, which may affect the expression level and transcription regulation pattern of hR. Washing the KM-1 membrane with a low ionic strength solution produced an hR-enriched membrane, which showed a Cl^- binding absorption shift similar to that of solubilized and purified *N. pharaonis* hR.

2. Materials and methods

2.1. Strains and growth conditions

The type strain of *N. pharaonis* JCM 8858^T (DSM 2160^T) was obtained from the culture collection Japan Collection of Microorganisms (JCM) (RIKEN, Japan). One liter of the culture medium (pH 9.0)

*Corresponding author. Fax: +81 52 789 4526.
E-mail address: ihara@gene.nagoya-u.ac.jp (K. Ihara).

Abbreviations: N. pharaonis, *Natronomonas pharaonis*; *H. salinarum*, *Halobacterium salinarum*; bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; pR, phoborhodopsin; bat, bacteriorhodopsin activator; hop, haloopsin; bop, bacterioopsin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin layer chromatography; OD, optical density; ORF, open reading frame

0014-5793/$34.00
doi:10.1016/j.febslet.2008.07.030

contained: 200 g NaCl, 1 g KCl, 1 g NH_4Cl, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g casamino acids (Becton–Dickinson, USA), 5 g Na_2CO_3, and trace metals (0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.03 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg H_3BO_3, 0.2 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuCl_2 \cdot 2H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, and 0.03 mg $Na_2MoO_4 \cdot 2H_2O$). Cells collected from single colonies grown on agar were transferred to culture tubes containing 3 ml liquid medium and shaken at 40 °C for 3 days. The cells were then transferred to 500 ml Erlenmeyer flasks containing 100 ml medium, and when they reached the late-exponential phase, they were inoculated into 8 l medium in a 10-l glass culture container.

2.2. Membrane isolation

The cells were centrifuged at $10000 \times g$ for 15 min, and the pellet was suspended in buffered salt solution A (3 M NaCl, 50 mM HEPES–NaOH, pH 7.0) and frozen at −80 °C until use. The frozen cells were thawed using flowing tap water. To reduce the viscosity of the suspension, DNase (1 mg aliquot for 10 g wet cells) and Mg^{2+} (final concentration 5 mM in the form of chloride salt) was added to the suspension followed by stirring for 60 min at room temperature. The suspension was centrifuged at $10000 \times g$ for 15 min at 4°C to remove undisrupted cells, and the supernatant was centrifuged at $90000 \times g$ for 60 min at 4 °C. The precipitated membrane fraction was washed once with solution A and then three times with buffered salt solution B (100 mM NaCl and 50 mM HEPES–NaOH, pH 7.0). For the chloride ion titration experiment, the membrane was further washed three times with distilled water and then washed by 0.2% (w/v) Tween 20 and finally suspended in distilled water.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and thin layer chromatography (TLC) analysis of the membrane

Gel electrophoresis was performed in a 2-mm flat gel in 15% acrylamide at constant concentration, according to the procedure of Laemmli [11]. Total lipids were extracted using the Bligh and Dyer method [12], as modified for extreme halophiles by Kates. Pigments were analyzed using TLC on silica gel 60 F_{254} plates (Merck, Germany).

2.4. Spectroscopic measurements

Absorption spectra were measured using a UV-2500 Shimadzu spectrophotometer (Shimadzu, Japan). The obtained spectral data were further analyzed using Microsoft Excel (Microsoft Co., USA) and Origin (OriginLab, USA) softwares.

2.5. Flash photolysis spectroscopy experiment

Samples were excited at 532 nm using the second harmonic of the fundamental band of Nd-YAG laser (Quantel Ultra, France). The source of the monitoring light was a Xenon arc lamp (Hamaphotonics E7536 Hamamatsu photonics, Japan) and the beam of the monitoring light was perpendicular to that of actinic flash. At each wavelength, 500 laser pulses were averaged to improve signal-to-noise ratio. The apparatus and procedure of flash-photolysis in details will be described elsewhere (Hayakawa, N. et al., in submission).

2.6. Genomic DNA isolation and sequencing

Genomic DNA was isolated from the wild-type strain of *N. pharaonis* and the mutant strain KM-1, according to the method of Marmur [13]. Its purity was assessed based on the A_{260}/A_{280} and A_{230}/A_{260} extinction ratios. The regions coding the haloopsin (*hop*) and *bat* genes of *N. pharaonis* were amplified using the polymerase chain reaction (PCR) method with the following primers: hop_f, 5′-GATGAGAACGGCGGCAACCAGAAA; hop_r, 5′-GTTTCTGGCTCTACCCGGACCAAA; bat_f, 5′-CTGAACCGACGCCCGCCTTTATTT; and bat_r, 5′-CCGTGCGTTGGGATGTCGAGATTA. After purifying the PCR products using a PCR purification kit (Promega, Japan), the *hop* and *bat* gene-encoding regions were directly sequenced with primers (see Supplement for details) using the Big-Dye terminator ver.3 kit (ABI, USA) and ABI sequencer 3100 (ABI, USA).

2.7. Isolation of RNA and transcript analysis

Total RNA was isolated from *N. pharaonis* cultures at optical density $(OD)_{680} = 0.1–1.0$, according to the Chomczynski and Sacchi method [14]. Northern analyses involved electrophoresis of 1.0 μg RNA on denaturing formaldehyde-containing 1.0% agarose gels, followed by capillary transfer to nylon membranes. A double-strand DNA probe corresponding to the *N. pharaonis hop* open reading frame (ORF) region was amplified using the PCR method with the following primers: *hop*_ORFf, 5′-ATGACTGAGACATTGCCACCGGTA and *hop*_ORFr, 5′-TCAGTCGTCAGCGGGAGTGCCCGA. The DNA probe was labeled with alkaline phosphatase using AlkPhos Direct Labeling Kit (GE Healthcare, Sweden). Northern hybridization was performed according to the manufacturer's instruction: hybridization was performed at 55 °C at a probe concentration of 10 ng/ml, followed by washing twice at 55 °C. CDP-star chemiluminescence was used for the detection of the position of probe binding.

3. Results

Of the 10^4 colonies of *N. pharaonis* grown after ultraviolet (UV) irradiation, a single purplish colony was selected from the remaining orange wild-type colonies and was completely segregated using three cycles of inoculation. In the dark condition, the growth of the mutant strain KM-1 was similar to that of the wild-type strain but was slightly inhibited in the light condition (data not shown). To investigate the origin of the purple color in strain KM-1 (Fig. 1A), membrane fractions were prepared, and their protein and pigment components were analyzed using SDS–PAGE and TLC, respectively.

The membranes prepared from the wild-type strain and strain KM-1 were analyzed using SDS–PAGE (Fig. 1B). In *N. pharaonis*, stable membrane fractions were recovered after

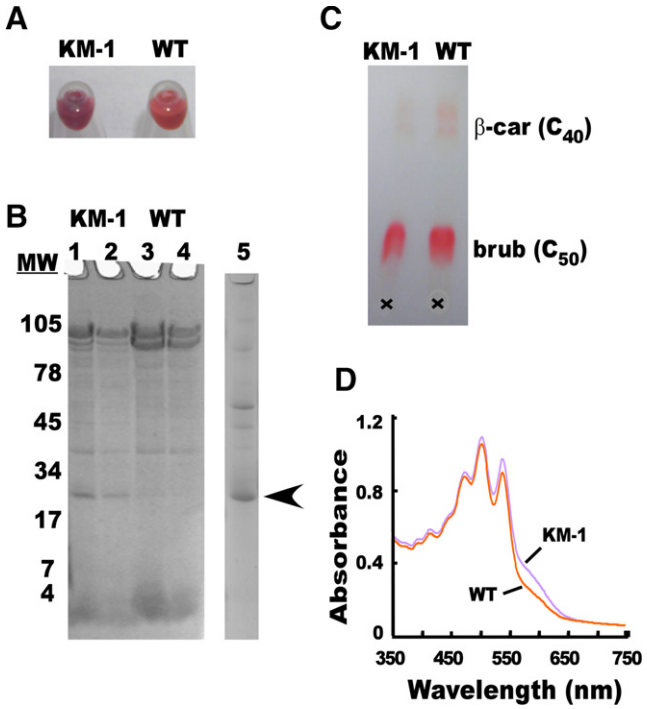

Fig. 1. (A) Cell pellets of *N. pharaonis* hR-overexpressing strain KM-1 (left) and wild-type strain (right). (B) SDS–PAGE of the membranes isolated from strain KM-1 (lanes 1 and 2), the wild-type strain (lanes 3 and 4) and Tween washed membrane from strain KM-1 (lane 5); lanes 1 and 3, 30 μg protein/lane and lanes 2 and 4, 10 μg protein/lane. (C) TLC analysis of chloroform–methanol (1:2) extracts from strain KM-1 (left) and wild-type (right). Spotted positions are marked as crosses (X); the positions of β carotene and bacterioruberin are indicated as β-car (C_{40}) and brub (C_{50}), respectively. (D) Absorption spectra of the membranes isolated from strain KM-1 and the wild-type strain.

washing with a low ionic strength solution that did not contain magnesium ions. The membranes contained 100-kDa glycoproteins, which was confirmed using the glycoprotein-staining method (data not shown). In strain KM-1, a protein band of approximately 25 kDa was prominently increased (50-fold) and its fraction of the total protein was estimated to be 0.2, by densitometric measurement. A large fraction of glycoproteins was removed by treating the membrane with 0.3% (w/v) Tween 20 and the hR fraction of the total protein was estimated to be 0.65 (Fig. 1B, lane 5). Next, $CHCl_3$/MeOH (1:2) extracts were analyzed using TLC (Fig. 1C). In both strains, the red pigment corresponding to C_{50} bacterioruberin formed a major band, which was deduced from a similar retardation factor value with the bacterioruberin extracted from the authentic standard cell *H. salinarum*. Small amounts of the orange bands corresponding C_{40} β-carotene were also detected. The amount of bacterioruberin was almost the same in the two strains, but the amount of β-carotene in strain KM-1 was one-third that in the wild-type strain (Fig. 1C).

The absorption spectra of membrane suspensions prepared from the wild-type strain and strain KM-1 were very similar in the range 400–550 nm, where the characteristic peaks at 474, 504, and 540 nm originating from bacterioruberin were observed (Fig. 1D). The most prominent difference was the shoulder at approximately 600 nm in the strain KM-1 membrane (Fig. 1D). The shoulder spectra drastically changed with the chloride concentration (Fig. 2A), which strongly suggested that the absorption originated from the retinal protein hR. Experimental data are best fitted by the equation $\Delta A/\Delta A_{max} = 1/[1 + ([Cl^-]/K_m)^n]$ with the dissociation constant $K_m = 1.7$ mM and the cooperative index $n = 0.7$. A similar binding constant for chloride ion ($K_m = 1$ mM) and negative cooperativity for the binding ($n = 0.75$) was observed for the hR membrane produced by the heterologous expression systems [15]. The chloride ion dependent change of absorption spectra strongly indicated that the overexpressed 25-kDa protein was hR. Furthermore, light-induced absorption changes of the membrane at 520, 580, 600, and 640 nm were investigated in the 2 M NaCl or the 2 mM NaCl solutions (Fig. 3). The results were completely explained by the *N. pharaonis* hR photocycle, whose scheme was reported to be $hR_{575} \rightarrow K_{570} \rightarrow L(N)_{520} \rightarrow O_{600} \rightarrow hR_{575}$ [16]. The O_{600} kinetics strongly depended on the chloride ion concentration (Fig. 3). These photochemical features of the membrane strongly indicated that the strain KM-1 is hR-overproducing mutant from *N. pharaonis*.

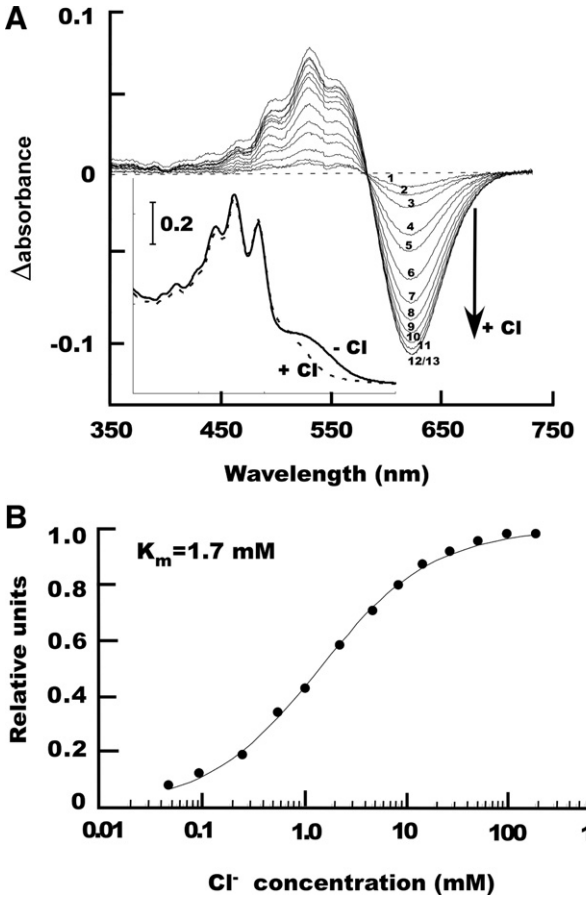

Fig. 2. (A) Difference spectra of the hR-containing membrane obtained from strain KM-1, titrated using NaCl. The chloride concentrations were: (1) 0.05 mM, (2) 0.1 mM, (3) 0.27 mM, (4) 0.60 mM, (5) 1.10 mM, (6) 2.42 mM, (7) 5.07 mM, (8) 9.05 mM, (9) 15.65 mM, (10) 28.78 mM, (11) 54.80 mM, (12) 105.82 mM, and (13) 204.0 mM. The inset shows the absorption spectra in the absence (bold line) and presence (dotted line) of chloride ions. (B) Amplitudes of the difference spectra with and without chloride vs. the logarithm of chloride concentration. The solid line was calculated using a reaction order of chloride ions of 0.75 and a binding constant of 1.7 mM.

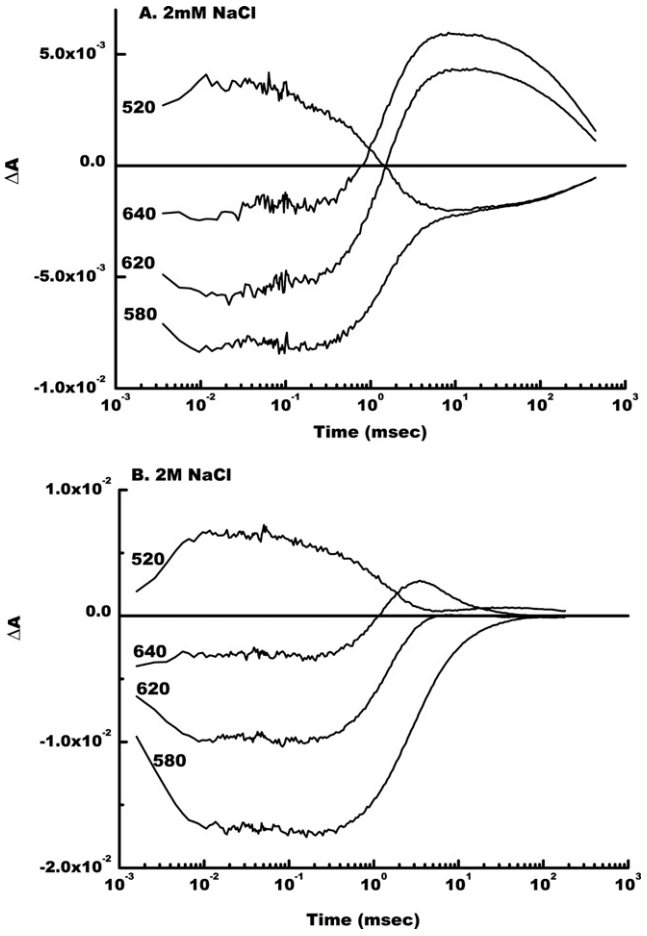

Fig. 3. Traces of the transient absorption change after photoexcitation of the Tween washed KM-1 membrane in the presence of 2 mM NaCl (A) or in the presence of 2 M NaCl (B). Time courses of selected four wavelengths (520, 580, 620, and 640 nm) are shown.

In order to investigate hR overexpression in strain KM-1, the transcription levels of the *hop* gene at various growth stages (early, mid, late exponential, and stationary phases) were investigated using northern hybridization analyses (Fig. 4). In the wild-type strain, the *hop* transcript was not detected in the early to mid-exponential phases and was detected only after the late-exponential phase (Fig. 4B). This induced transcription pattern of the *hop* gene in *N. pharaonis* was similar to the pattern of *bop* gene expression in *H. salinarum*. Interestingly, the transcription of the *hop* gene in strain KM-1 changed to the constitutive pattern when its expression level increased (Fig. 4C). The overexpression of hR in strain KM-1 may be primarily attributable to a change in transcription regulation.

Because the amounts of transcription of the hop gene and its expression pattern were completely changed in strain KM-1, the *hop* gene sequence, including 5′ and 3′ flanking regions, was completely sequenced and compared with that of the wild-type strain. No changes in the nucleotides, including the putative promoter region nucleotides, were observed between the two strains (see Supplement for details). In *H. salinarum*, the *bop* gene expression was primarily regulated by bat, and mutations in *bat* are known to constitutively alter the expression of the *bop* gene. In *N. pharaonis*, a *bat* gene homologue was found 7.2 kb upstream of the *hop* gene. The complete sequence of the *bat* gene from strain KM-1 revealed a point mutation (G–A) at the 970th nucleotide, which caused an amino acid change from aspartic acid to asparagine. This point mutation was a candidate mechanism for the constitutive expression of the *hop* gene in the mutant strain.

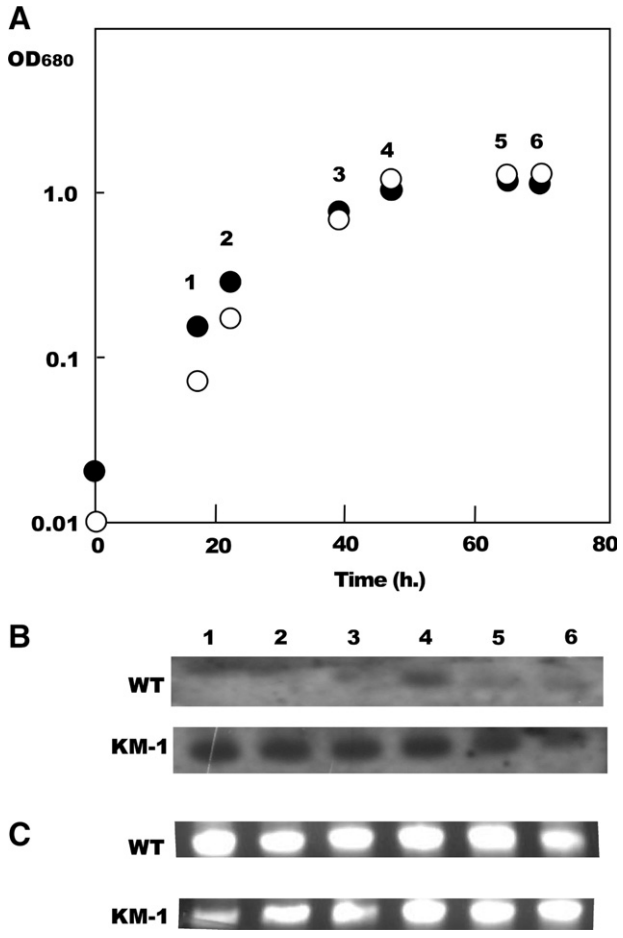

Fig. 4. (A) Growth curve of *N. pharaonis*: wild-type strain (open circle) and strain KM-1 (closed circle). Samples (5 ml) were withdrawn from each growth medium at 18 h (1), 24 h (2), 40 h (3), 48 h (4), 68 h (5), and 72 h (6) after inoculation, and growth was measured based on the OD at 680 nm. (B) The *hop* gene transcription levels at each growth point (1–6). Each sample (3 μg RNA) was loaded on a 1.0% denatured agarose gel and transferred to a nylon membrane. See Section 2 for details. (C) The amount of 16S rRNA at each growth point (1–6).

4. Discussion

In this study, we selected the hR-overexpression mutant strain KM-1, which was produced using UV mutagenesis of the type strain of *N. pharaonis* (DSM 2160^T). The expression level of hR was estimated to be several tens of times of that in the wild-type strain and was almost equal to the expression level of bR in *H. salinarum*. This implied that the mutant strain was a good source of authentic hR in its natural lipid environment for use in various physicochemical measurements, including structure analysis, and other applications.

H. salinarum can synthesize ATP in the presence of light by using bR and hR [17,18]. In the presence of light, bR sustained prolonged ATP synthesis, while hR maintained maximum ATP levels for only several minutes due to intercellular acidification [19]. Therefore, hR does not function as a part of the physiological light-driven ATP synthesis machinery, at least in the strain *H. salinarum*. While, hR-mediated ATP synthesis was sustained for a long time in the strain *N. pharaonis* (personal communication from Prof. Martin Engelhard and our unpublished data). Because of this feature for the haloalkaliphiles, natural hR-overexpression mutant was successfully obtained from the *N. pharaonis*.

The membranes of *N. pharaonis* were rather stable even in a low ionic strength solution that did not contain divalent cations. *N. pharaonis* was originally discovered in conditions of low Mg^{2+} concentration; further, it retained its rod-shaped morphology at alkaline pH. Therefore, the mechanism of the maintenance of the S-layer in *N. pharaonis* cells must differ from that in the halophilic archaea living at neutral pH; the latter were easily transformed to spheroplasts by the chelation of Mg^{2+} by using ethylene diamine tetraacetic acid [20]. After washing the membrane with distilled water, hR-enriched membranes were obtained; these membranes retained carotenoids. Furthermore, unlike the purple membrane of *H. salinarum*, no two-dimensional crystalline array of hR was formed in the isolated membrane (Narusawa et al., unpublished data). In fact, the hR-enriched membranes were composed of several proteins, including 100-kDa glycoproteins other than hR. The *H. salinarum* cell membrane contains some glycolipids that were known to be localized in the purple membrane [21]. While no glycolipids were present in the *N. pharaonis* cell [2], several glycoprotein-encoding genes were found in the genome [3]. This might be responsible for the difference in the homogeneity and/or stability of the membranes of *H. salinarum* and *N. pharaonis* under low-salt conditions.

From the *Halorubrum* spp. aus-1 and aus-2, claret-colored membranes were isolated, which were similar to the purple membrane but contained bacterioruberin as a second pigment [22–25]. Structure analyses with trigonal and hexagonal lattice crystals prepared using the membrane-fusion method revealed that the bacterioruberin molecule binds to a crevice between

adjacent subunits in the trimer structure contributing to stabilization of archaerhodopsin-2 in the claret membrane [26]. The absorption spectra were measured using dried film of claret membrane from *N. pharaonis*. These films were inclined at an angle of 45° from the propagation direction of the polarized light used for measurement. The absorption spectra thus obtained were very similar to those of the claret membrane obtained from *Halorubrum* sp. aus-2 (Narusawa and Kouyama, unpublished data). These data suggest that bacterioruberin plays a similar function in the stabilization of the hR structure in the claret membrane.

Scharf and Engelhard reported that the hR content of *N. pharaonis* increased at low oxygen concentrations [27]. Our results regarding *hop* gene transcription in the wild-type strain were consistent with their observation. In the wild-type strain of *N. pharaonis*, the *hop* gene was expressed from the late exponential to stationary phases, during which the oxygen concentration was decreased. This expression pattern closely resembles the pattern of *bop* gene expression in *H. salinarum* [18]. In this context, hR may function as an ATP synthase driver, similar to bR, acting as an auxiliary engine under anaerobic conditions in *N. pharaonis*. In *H. salinarum*, *bop* gene expression was regulated by the activator gene *bat* [28,29], which also controls the transcription of some other genes related to the biosynthesis of carotenoids and retinal [30]. The β-carotene concentration in strain KM-1 was reduced (Fig. 1D), which might be a result of the upregulation of the β-carotene cleavage enzyme brp [31]. Interestingly, these genes concerning bR biosynthesis were also found in the region proximal to the location of the *hop* gene in the *N. pharaonis* genome. Because the *hop* gene transcription level in *Halobacterium* was not affected by *bat* gene mutation or disruption [30], the regulated expression of hR might be a unique characteristic of *N. pharaonis* or of extremely haloalkaliphilic archaea. In order to confirm this, we should establish a transformation system in haloalkaliphilic archaea and introduce site-directed mutations in the *bat* gene that alter hop gene expression.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.030.

References

[1] Soliman, G.S.H. and Trüper, H.G. (1982) *Halobacterium pharaonis* sp. nov., a new extremely haloalkaliphilic archaebacterium with low magnesium requirement. Zbl. Bakt. Hyg. I Abt. Orig. C 3, 318–329.

[2] Tindall, B.J., Ross, H.N.M. and Grant, W.D. (1984) *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. Syst. Appl. Microbiol. 5, 41–57.

[3] Falb, M., Pfeiffer, F., Palm, P., Rodewald, K., Hickmann, V., Tittor, J. and Oesterhelt, D. (2005) Living with two extremes: conclusions from the genome sequence of *Natronomonas pharaonis*. Genome Res. 15, 1336–1343.

[4] Duschl, A., Lanyi, J.K. and Zimanyi, L. (1990) Properties and photochemistry of a halorhodopsin from the haloalkalophile, *Natronobacterium pharaonis*. J. Biol. Chem. 265, 1261–1267.

[5] Hirayama, J., Imamoto, Y., Shichida, Y., Kamo, N., Tomioka, H. and Yoshizawa, T. (1992) Photocycle of phoborhodopsin from haloalkaliphilic bacterium (*Natronobacterium pharaonis*) studied by low-temperature spectrophotometry. Biochemistry 31, 2093–2098.

[6] Scharf, B. and Wolff, E.K. (1994) Phototactic behaviour of the archaebacterial *Natronobacterium pharaonis*. FEBS Lett. 340, 114–116.

[7] Bivin, D.B. and Stoeckenius, W. (1986) Photoactive retinal pigments in haloalkaliphilic bacteria. J. Gen. Microbiol. 132, 2167–2177.

[8] Wagner, G., Oesterbelt, D., Krippahl, G. and Lanyi, J. (1981) Bioenergetic role of halorhodopsin in *Halobacterium halobium* cells. FEBS Lett. 131, 341–345.

[9] Heymann, J.A., Havelka, W.A. and Oesterhelt, D. (1993) Homologous overexpression of a light-driven anion pump in an archaebacterium. Mol. Microbiol. 7, 623–630.

[10] Havelka, W.A., Henderson, R., Heymann, J.A. and Oesterhelt, D. (1993) Projection structure of halorhodopsin from *Halobacterium halobium* at 6 Å resolution obtained by electron cryomicroscopy. J. Mol. Biol. 234, 837–846.

[11] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

[12] Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

[13] Marmur, J. and Doty, P. (1961) Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. 3, 585–594.

[14] Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

[15] Varo, G., Brown, L.S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995) Light-driven chloride ion transport by halorhodopsin from *Natronobacterium pharaonis*. 1. The photochemical cycle. Biochemistry 34, 14490–14499.

[16] Chizhov, I. and Engelhard, M. (2001) Temperature and halide dependence of the photocycle of halorhodopsin from *Natronobacterium pharaonis*. Biophys. J. 81, 1600–1612.

[17] Matsuno-Yagi, A. and Mukohata, Y. (1980) ATP synthesis linked to light-dependent proton uptake in a red mutant strain of *Halobacterium* lacking bacteriorhodopsin. Arch. Biochem. Biophys. 199, 297–303.

[18] Oesterhelt, D. and Stoeckenius, W. (1973) Functions of a new photoreceptor membrane. Proc. Natl. Acad. Sci. USA 70, 2853–2857.

[19] Mukohata, Y. and Kaji, Y. (1981) Light-induced ATP synthesis dependent on halorhodopsin-pH regulation. Arch. Biochem. Biophys. 208, 615–617.

[20] Mescher, M.F. and Strominger, J.L. (1976) Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium*. Proc. Natl. Acad. Sci. USA 73, 2687–2691.

[21] Essen, L., Siegert, R., Lehmann, W.D. and Oesterhelt, D. (1998) Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin–lipid complex. Proc. Natl. Acad. Sci. USA 95, 11673–11678.

[22] Mukohata, Y., Ihara, K., Uegaki, K., Miyashita, Y. and Sugiyama, Y. (1991) Australian halobacteria and their retinal-protein ion pumps. Photochem. Photobiol. 54, 1039–1045.

[23] Uegaki, K., Sugiyama, Y. and Mukohata, Y. (1991) Archaerhodopsin-2, from *Halobacterium* sp. aus-2 further reveals essential amino acid residues for light-driven proton pumps. Arch. Biochem. Biophys. 286, 107–110.

[24] Sugiyama, Y., Maeda, M., Futai, M. and Mukohata, Y. (1989) Isolation of a gene that encodes a new retinal protein, archaerhodopsin, from *Halobacterium* sp. aus-1. J. Biol. Chem. 264, 20859–20862.

[25] Mukohata, Y., Sugiyama, Y., Ihara, K. and Yoshida, M. (1988) An Australian halobacterium contains a novel proton pump retinal protein: archaerhodopsin. Biochem. Biophys. Res. Commun. 151, 1339–1345.

[26] Yoshimura, K. and Kouyama, T. (2008) Structural role of bacterioruberin in the trimeric structure of archaerhodopsin-2. J. Mol. Biol. 375, 1267–1281.

[27] Scharf, B. and Engelhard, M. (1994) Blue halorhodopsin from *Natronobacterium pharaonis*: wavelength regulation by anions. Biochemistry 33, 6387–6393.

[28] Gropp, F. and Betlach, M.C. (1994) The bat gene of *Halobacterium halobium* encodes a trans-acting oxygen inducibility factor. Proc. Natl. Acad. Sci. USA 91, 5475–5479.
[29] Leong, D., Pfeifer, F., Boyer, H. and Betlach, M. (1988) Characterization of a second gene involved in bacterio-opsin gene expression in a halophilic archaebacterium. J. Bacteriol. 170, 4903–4909.
[30] Baliga, N.S. et al. (2002) Coordinate regulation of energy transduction modules in *Halobacterium* sp. analyzed by a global systems approach. Proc. Natl. Acad. Sci. USA 99, 14913–14918.
[31] Peck, R.F., Echavarri-Erasun, C., Johnson, E.A., Ng, W.V., Kennedy, S.P., Hood, L., DasSarma, S. and Krebs, M.P. (2001) *brp* and *blh* are required for synthesis of the retinal cofactor of bacteriorhodopsin in *Halobacterium salinarum*. J. Biol. Chem. 276, 5739–5744.